REMARKS

Examiner is thanked for the Official Action of 2009.06.01. This request for reconsideration is intended to be fully responsive thereto.

CLAIM AMENDMENT

Claim 1 has been amended based on Example 1 (however, an adenovirus is used also in transferring a first recombinant DNA) and Example 2 in the specification (see page 21, line 2 to page 36, line 12 of the specification and FIG. 1).

Claim 2 has been amended based on Example 3 in the specification (see page 36, line 13 to page 42, line 14 in the specification and FIG. 1).

Amendment to Claims 1 and 2 has been made based on the disclosure of the specification in conformity with the requirement defined in USC §112, second paragraph, and thus does not introduce new matter.

CLAIM REJECTIONS UNDER 35 USC §103

The Examiner states that Claims 1-4, 6, 7, 14-18, 33, 34 and 36 remain rejected under 35 USC §103 as being unpatentable Vallier *et al.* in view Ong *et al* and Rybkin *et al.* and further in view of Yamamoto *et al.*.

The present invention does not seem to be self-evident from the citations indicated by the Examiner, and the reason for this will be explained below.

(1) The background based on which the present invention had been accomplished will be explained in detail below.

(a)The Examiner states that "none of the citations disclose the same invention as the present invention," indicating four citations, Vallier *et al.*, Ong *et al.*, Rybkin *et al.* and Yamamoto *et al.*

However, "the 'problem,' which was difficult to solve by means of the prior art, but was solved by the present invention for the first time, itself, had not been recognized at the time when the invention was accomplished." Further, "those skilled in the art, of course, had no consciousness of the problem as having not recognized the problem, and thus had not pursued the cause thereof. Further, there is no document revealing a scientific "cause" for the failure of the prior art to function, and thus the 'cause' was completely unknown at the filing date of the present application." Namely, although the problem to be solved by the present invention and the cause of the problem "had been clarified for the first time in the present specification," the Examiner misunderstands that the problem and the cause were already known, and thus concludes that the present invention would be obvious. That is, it seems that the Examiner somewhat misunderstands as if the premises (the "problem" to be solved and the scientific "cause" thereof) were commonly known *per se* at that time, and thus erroneously concludes that the present invention could have been easily made by researchers in the research field at the filing date of the present application by combining four citations completely irrelevant to each other (these citations merely individually disclose some of the parts, are different in the "object" from the present invention, and nowhere disclose the "solving means" constituting the basis of the present invention). Needless to say, "the idea/action of forcibly combining four documents completely irrelevant to each other could never occur" if "there is no problem to be solved that constitutes the basis of the present invention, namely, no 'trigger'".

While the present invention relates to the technique of "identifying and isolating a target cell differentiated from an ES (embryonic stem) cell," the only one conventional method involves "establishing an ES cell line wherein a light emitting protein is directly expressed with a tissue-specific promoter (more specifically, preparing a gene construct

wherein a light emitting protein is directly expressed with a tissue-specific promoter, transferring the gene into the cell, selecting out an ES cell line wherein the gene is stably expressed, and further selecting out a cell line functioning correctly)." However, as described in the present specification, the inventors attempted to identify and isolate a cardiac muscle cell using this prior art, but the cell did not function at all. No document (neither patent documents, nor scientific documents) has ever been reported before, which presents the so-called negative result that "the only one prior art does not function in this manner," and at least review articles in this technical field at that time, though reviewed carefully, nowhere report such negative results. In view of these, there is no fact that the problem with the prior art was commonly known to researchers in the technical field. Accordingly, since the problem had not been recognized as the problem to be overcome, "the 'scientific cause' of the failure of the only one prior art to function" had neither been sought, nor, of course, been found out before. The scientific cause is that "the activity of the tissue-specific promoter has not reached the level ensuring the visualization of the expression, in a target cell differentiated from an ES cell, of a light emitting protein directly linked to the downstream thereof to such a level that the cell can be identified and isolated." This fact had been found out by the present inventors for the first time in the course of the present invention as will be stated below, and is explicitly described as a Comparative Example in the specification. Hereinafter, this point will be explained in more detail.

(b) When visualizing and isolating a target cell differentiated from an ES (embryonic stem) cell, the "only one" conventional method, for most of cell species having no cell (tissue)-specific surface protein marker, requires enormous operations requiring time and labor, namely,

1) preparing a gene construct wherein a promoter for a gene expressed specifically in the target cell (a tissue-specific promoter, what is called) is directly linked

to a light emitting protein gene as a marker;

2) transferring the gene construct into the ES cells, so that gene incorporation occurs in the cells into which the gene construct has correctly been introduced, more specifically, in the chromosomes, and selecting and isolating a part of the cells in which the gene construct is stably expressed (most of the cells do not cause gene incorporation in their chromosomes, and the transferred gene would disappear within several days) as clones; and

3) further reviewing and selecting cells wherein the gene construct of interest is correctly expressed and functions (in this case, when the ES cells have been differentiated into the target cells, the target light emitting molecules are expressed only in the target cells to ensure the visualization and isolation of the target cells) from these selected clone cells.

The present inventors also firstly attempted to visualize and isolate a target cell differentiated from an ES cell by this "only one" conventional method "requiring enormous time and labor," and, as a result, found out, "for the first time," that the case where "the target cell cannot be visualized by the conventional method" could occur "generally" (general phenomenon in any site not limited to the heart), "at high probability," and, additionally, "completely unpredictably" for the uncertainty thereof (see Comparative Examples in the present specification, pages 15 to 21). Namely, even the "fact" that "the 'only one' conventional method fails to function" had hitherto been completely unknown, and thus, of course, the "reason" why the conventional method does not function (as will be stated below, the expression at the level ensuring the visualization of a light emitting gene cannot be obtained by the conventional method for directly linking a tissue-specific promoter to a light emitting protein gene, namely, the conventional method does not function, in many cases, due to the weak activity of the tissue-specific promoter) had also been completely unknown in the past. "This

problem had not conventionally been known as a problem to be solved by a novel invention," but "had been found out by the present invention for the first time."

As for this fact, detailed experiments were conducted in the present invention, and the results are described as comparative experiments in the specification (page 15, line 2 to page 21, 1st line). Namely, using a Nkx2.5 gene promoter already confirmed to ensure cardiac muscle-specific expression (about 3kb of the upstream of the Nkx2.5 gene) (page 15, line 20 to page 16, line 4), a gene construct recombined to express an EGFP gene, as a light emitting protein gene, and a drug-resistant Neo gene was transferred into an ES cell under the Nkx2.5 gene promoter by this "only one" conventional method, and then, drug resistance performance was utilized to isolate and collected 78 clones therefrom (page 17, lines 19 to 20). However, among these clones, the number confirmed to have a correct gene construct introduced therein by two PCR assays were thirteen (page 18, lines 6 to 13). Thus, the thirteen clones were used (for reliable verification with generality and reproducibility) to carry out experiments in two different cardiac muscle cell differentiating systems, and then the following similar results were obtained and verified in the two experimental systems. Namely, the ES cells were surely differentiated into cardiac muscle cells (cardiac muscle-specific gene expression was confirmed, and pulsation particular to cardiac muscle cells was seen in the ES cells). Further, by the very sensitive RT-PCR method, the result was obtained that the expression of mRNA of the EGFP gene in the ES cells was also observed, but that "the EGFP protein was not obtained at such a level that it could be identified as a light emitting cell" with a fluorescent microscope or a cell sorter (page 19). In this way, the present inventors had found out, for the first time, that the conventional method for visualizing and isolating a target cell differentiated from an ES cell involves a serious problem.

Then, the present inventors, as a result of repeated reviews about this problem,

had revealed that "the low activity of the tissue-specific promoter for use in the visualization and isolation of a target cell is the 'cause'" (page 44, first line to page 45, first line, and FIG. 7). Since "it had hitherto not been revealed even that the failure of the only one conventional method to function is problematic," it is, of course, self-evident that "none of researchers noticed or found out this cause." Actually, none of documents and books in the field of stem cell biology disclose to that effect.

Then, the present inventors, as a result of various reviews about solutions based on this finding, had accomplished the present invention ensuring the visualization and isolation of an ES cell expressing a tissue specific promoter with a constitutive strong expression promoter.

The present invention had found out the problem associated with the only one conventional method for visualizing and isolating a target cell, and further had discovered the cause of this problem as stated above, and thus the advantageous effects thereof are quite remarkable. In fact, the technique of the present invention appeared, as the variable research contributing to the stem cell biology, in Molecular Therapy, which is an official journal of "American Society of Gene and Cell Therapy" as one of the most authoritative academic institutes in the field of gene therapy and cell therapy, and, additionally, appeared in the News column in "In this month" (important article in this number) in the same number of the same journal, and, besides, appeared on the cover (meaning the most important article in this number). (See attached reference) Namely, this fact clearly indicates that it was difficult for researchers having sufficient techniques and knowledge in the same field to accomplish the present invention at that time (even at the time when the present invention appeared in the journal, several years after the filing date of the present invention), and thus that they could never accomplished the present invention even by gathering the prior art documents (that merely describes some of the parts, and are completely different in

object from the present invention) cited by the Examiner.

In addition, the present invention is not only quite useful in the regeneration medicine, embryology and other molecular biology studies utilizing the differentiation of ES cells, but also quite useful in the future development of regeneration medicine for various inveterate diseases including cardiac infarct and cerebral infraction using these target cells, and the advantageous effects thereof are quite remarkable. Furthermore, the method as claimed in Claim 2 provides the particularly remarkable effects that it does not require any troublesome procedures of collecting an ES cell clone, and thus can save the time and labor. On the contrary, the conventional procedures are a quite uncertain method which requires enormous time and labor, but does not function, as the present inventors demonstrated and state in detail in the specification as discussed above.

(2) The Examiner states that Vallier et al discloses a method of gene trap comprising transfection with two recombinant vectors, a first and a second recombinant vector. Additionally, quantification of EGFP-expressing undifferentiated embryonic stem cells and there derivatives was performed by FACS.

However, Vallier *et al.* relates to the development of a drug-inducible system for expressing a differentiation-induced gene to be introduced only "after a target time" for the promotion of differentiation inducement of an ES cell (see Vallier *et al.*, page 2467, 2nd to 12th line from the bottom), merely uses the CAG (a constitutively activated promoter) and the Cre-loxP system as a means for attaining the object, and neither discloses nor suggests the present invention relating to a method for target cell-specifically enhancing the promoter activity to visualize and isolate the differentiation-induced target cell. Of course, neither discloses nor suggests the problem with the conventional method for visualizing and isolating a target cell differentiated from an ES cell.

The Examiner states that Ong et al is an exemplified prior art that teaches that it is routine or well-established in the art to use cell-type or tissue type restricted expression of certain promoters operatively associated with a recombinase gene in combination with a gene of interest that has been flanked by recombinase recognition sequences, wherein the recombinase is expressed under the control of the cell-type or tissue type specific promoter and when expressed results in the excision of the gene of interest.

However, Ong *et al.* neither discloses nor suggests the use of the Cre-loxP system to visualize and isolate an ES cell expressing a tissue-specific promoter with a constitutive strong expression promoter.

Further, Ong *et al.* neither discloses nor suggests the problem with the conventional method for visualizing and isolating a target cell differentiated from an ES cell.

The Examiner states that Rybkin et al teach inducible switch from proliferation to differentiation of mouse ventricular myocardium by conditional expression of simian virus 40 large T-antigen(Tag) under the control of the early cardiac promoter Nkx2.5 and Cre-mediated recombination.

However, Rybkin *et al.* neither discloses nor suggests the use of Nkx2.5 and Cre-recombinase to visualize and isolate an ES cell expressing a tissue-specific promoter with a constitutive strong expression promoter. Further, Rybkin *et al.* neither discloses nor suggests the problem with the conventional method for visualizing and isolating a target cell differentiated from an ES cell.

The Examiner states that Yamamoto et al successfully demonstrate conditional expression of the HST-1/FGF-4 gene in the testis of mice using the Cre/lox system by administration of a recombinant adenovirus expressing the Cre recombinase in vivo.

However, Yamamoto *et al.* involves the *in vivo* use of a Cre-expressing

adenovirus, and is different from the present invention involving the *in vitro* use of an adenovirus. Further, Yamamoto *et al.* neither discloses nor suggests the use of an adenovirus expressing Cre-recombinase to visualize and isolate an ES cell expressing a tissue-specific promoter with a constitutive strong expression promoter.

The present inventors had found out the problem associated with the conventional method for visualizing and isolating a target cell differentiated from an ES cell for the first time, had figured out the cause of the problem for the first time, and, additionally, had accomplished the means ensuring the visualization and isolation of the ES cell expressing a tissue-specific promoter with a constitutive strong expression promoter, and then had completed the present invention having quite remarkable advantages. Accordingly, those skilled in the art cannot arrive at the present invention even by combining, in any manner, Vallier *et al.*, Ong *et al.*, Rybkin *et al.* and Yamamoto *et al.* which neither disclose nor suggest the problem to be solved by the present invention.

None of Vallier *et al.*, Ong *et al.*, Rybkin *et al.* and Yamamoto *et al.* disclose or suggest the timing to transfer the first and second recombinant DNAs into ES cells and the timing for differentiation-inducement of the ES cells for the purpose of visualizing and isolating the ES cells with a constitutive strong expression promoter, and thus those skilled in the art cannot arrive at the present invention even by combining Vallier *et al.*, Ong *et al.*, Rybkin *et al.* and Yamamoto *et al.* in any manner.

CLAIM OBJECTIONS

The Examiner states that abbreviations such as CA in claim 4 should be spelled out at the first encounter in the claims.

Applicant amended claim to read "a hybrid promoter of cytomegalovirus enhancer and chicken β actin promoter". (See page 7, line 10-11)

The Examiner states that each of claims 15-17 refers to the product without referring to a preceding by use of a definite article "The" and the use of a definite article in this context is grammatically incorrect.

Applicant amended claim15-17 according to the Examiner's suggestion.

CLAIM REJECTIONS UNDER 35 USC §112-Second paragraph

Claim 1-4, 6,7,14-18, 30, 33, 34 and 36 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particular point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the limitation “strongly expressed by said first promoter” in claim1 is unclear how much the expression of the fluorescence protein is increased and relative to what.

Applicant amended claim 1 to remove "strongly expressed by said first promoter".

The Examiner states that the metes and bounds of "in this order" in claim 1 are indefinite.

Applicant amended claim 1 to remove "in this order".

The Examiner states that “a fluorescence protein of a target cell differentiated from an embryonic cell” in claim1 is unclear whether the fluorescence protein is selectively ……drive a fluorescence protein expression in the differentiated cell.

Applicant amended claim1 as attached.

The Examiner states that “with an adenovirus vector as an episomal form” in claim1 is unclear whether the first recombinant DNA and second recombinant DNA ……and the other is encoded by a different gene vector.

Applicant amended claim 1 as attached.

The Examiner states that the steps listed in the method is not apparent as to

under what structural or functional parameters the transferring of a first and a second recombinant DNA is indicative or correlative to the preamble of the claims.

Applicant amended claim1 as attached.

CONCLUSION

In view of the above, Applicant respectfully submits that each of Claims 1-4, 6,7,14-18, 21, 24, 27, 30, 33, 34 and 36 recites statutory subject matter that is novel and new, is subject matter of the present invention and is fully supported in the disclosure of the present invention, and therefore requested that Claims 1-4, 6,7,14-18, 21, 24, 27, 30, 33, 34 and 36 be found allowable and that this application be passed to issue. No new matter has been added.

If for any reason, the Examiner determines that the application is not now in condition for allowable, it is respectfully requested that the Examiner contact the Applicant's undersigned attorney at the indicated telephone number to arrange for an interview to expedite the disposition of this application.

Respectfully submitted,

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